GRAFT POLYMERIZATION VI. STUDIES ON PROOF OF GRAFTING AND MOLECULAR WEIGHTS OF THE ISOLATED POLY(METHYL METHACRYLATE) GRAFTS

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Abstract

Chromium-tanned sheepskins were grafted with methyl methacrylate by a redox initiation system. The grafted product was exhaustively extracted to remove homopolymer, enzymatically degraded to remove the bulk of the protein, solvent fractionated to obtain a soluble fraction, and chromatographed on a controlled-pore glass column. Viscosity studies and amino acid analyses of the resulting material indicated that there were from one to three amino acids left in the peptide attached to the synthetic polymer chain. A single peak was obtained from the chromatographic column; however, the polydispersity of the polymer is demonstrated by the average molecular weights of 1,218,000 for the first half of the peak and 559,000 for the remainder. Molecular weights of the extracted homopolymers were somewhat less than those of the graft copolymers and were affected by the duration and temperature of the solvent extraction.

Introduction

Previous studies (1–3) of the graft polymerization of vinyl monomers onto chromium-tanned sheepskins have shown that a number of monomers can be used to impart new properties to the finished leathers. Recent studies (4) with n-butyl acrylate have demonstrated that it is possible to isolate a high molecular weight polymer-peptide unit containing 2.83 percent amino acids on a weight basis. Viscosity studies of the final fractionated product indicated that the molecular weight was about one million.

In the present work we isolated polymer-peptide units from enzymatically degraded and solvent fractionated methyl methacrylate graft copolymer. We determined the molecular weight range of this grafted synthetic polymer and of the extracted unbound homopolymer by viscosity measurements. These results enabled us to establish that true grafting occurred between the collagen molecule and the synthetic polymer.

*Federal Research, Science and Education Administration, U. S. Department of Agriculture.

Experimental

Chemicals and Materials

1) Poly(methyl methacrylate) — Polysciences, Inc.*; poly(methyl methacrylate), secondary standard — Aldrich Chemical Company.

2) 2-Butanone (99+ percent) — Aldrich Chemical Company.

3) Controlled-Pore-Glass-10-1000 — Electro-Nucleonics, Inc.

4) Collagenase, *Clostridium histolyticum* — Aldrich Chemical Company.

5) Pepsin, hog stomach mucosa, 3X cryst. — Nutritional Biochemicals Company.

6) Pronase, B grade, 45,000 PUK/gm — Calbiochem.

7) Methyl methacrylate containing 10 ppm of the monomethyl ether of hydroquinone (MEHQ) as inhibitor — Rohm and Haas Company.

Other chemicals were obtained from a number of sources and used as received.

Graft Polymerization Procedure

A section of the chromium-III-tanned sheepskin (approximately 750 gm; 170 gm on a dry weight basis) was cut into several pieces which were placed in a one-gallon stainless steel drum. A 200 percent float (on a drained blue weight basis) was prepared with the following, based on the dry substance of the substrate: 4 percent potassium persulfate, 4 percent Triton X-100, and 0.28 percent sodium bisulfite. (*Note*: It was found necessary to prevent contact of the sodium bisulfite with the acid to avoid release of SO_2.) Sufficient dry ice was added to displace all the air, and the float was poured into the drum. A slow stream of nitrogen was bled in during the run while the mixture tumbled for 30 minutes at ambient temperature. At this point, methyl methacrylate was added on a 100 percent dry weight basis. (*Note*: The flammability and toxic nature of all the monomers required their handling with appropriate care.) Additional dry ice was added to maintain an oxygen-free atmosphere, and the mixture was tumbled as before. A slow stream of nitrogen was continued through the gudgeon during the 20 hours of tumbling. The product was then removed, washed thoroughly in cold running tap water, and allowed to dry at room temperature. The air-dried stock was cut into ¼-in. strips and ground three times in a Wiley Mill with a #10 screen.

Total Nitrogen Determination

The percent nitrogen was determined by the semimicro Kjeldahl method. These values were calculated on a moisture- and ash-free basis. The moisture was determined by drying the samples to constant weight in a vacuum oven at 50°C, and the ash values were obtained by heating the samples at 625°C for 2 hours.

*Reference to brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

Homopolymer Extraction

Approximately 3 gm of the air-dried, ground product was weighed into a thimble and extracted in a Soxhlet apparatus for 24 hr with ethyl acetate or acetone. This extract was dried on the steam bath and the residue was then heated for an additional 24 hr at 100°C, cooled, and weighed. If the extracted homopolymer was to be used for viscosity measurements, the solvent was evaporated under reduced pressure and room temperature. The polymer was further dried in a vacuum oven at 35°C for 48 hr. Drying at a higher temperature was avoided because it could result in "curing" of the polymer and thus interfere with the viscosity measurements. For the room temperature extractions, the thimbles were suspended in about 450 ml of the solvent. This was stirred constantly for 24 hr with a Teflon™ coated magnetic stirrer and then evaporated under reduced pressure as before. The ground graft copolymer was exhaustively extracted in a Soxhlet apparatus for three 24-hr periods to remove all homopolymer prior to degradation with the enzymes (Figure 1).

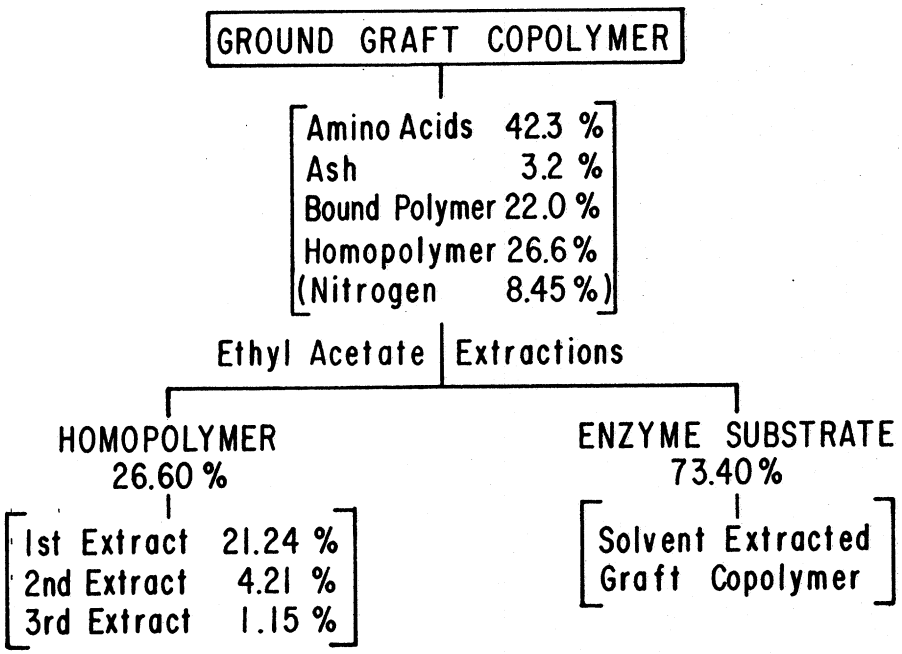

FIGURE 1.—Properties of methyl methacrylate graft copolymer.

Isolation of the Synthetic Graft

The air-dried, solvent-extracted sample (5.00 gm) in 100 ml of tris buffer (0.05 *M* tris hydroxymethylaminomethane containing 0.01 *M* $CaCl_2$ adjusted to pH 7.5 with 6 *N* HCl) (5) was stirred intermittently for 20 hr (Figure 2).

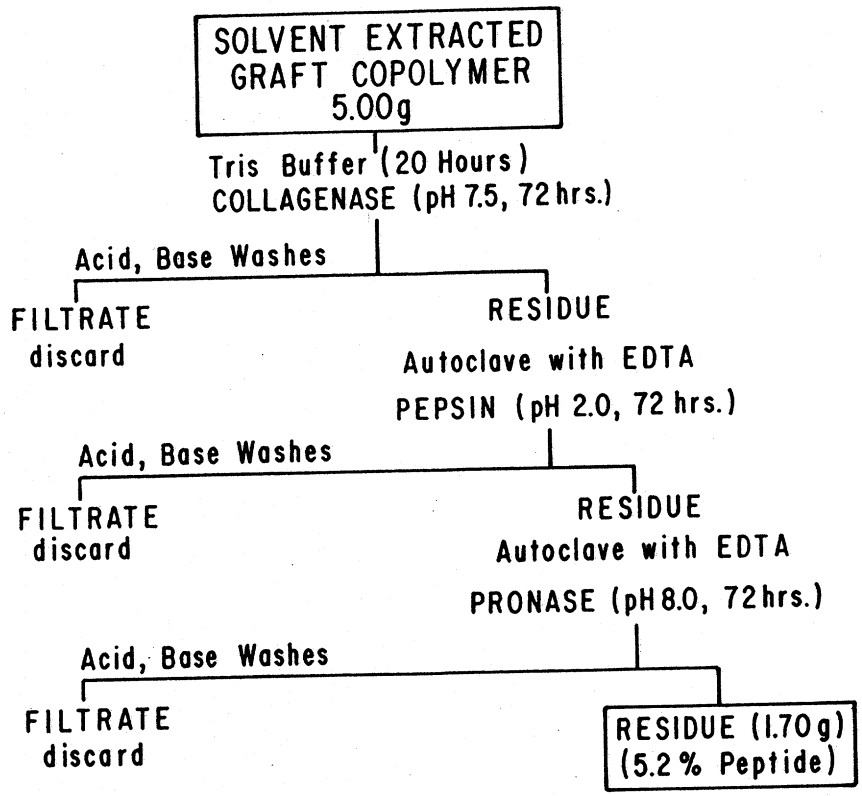

FIGURE 2.—Enzyme degradation procedure.

Collagenase (0.05 gm), was added and the sample was stirred intermittently for 72 hr. The sample was centrifuged to remove gelatinous material, then filtered through sintered glass, washed successively with 0.1 *N* HCl, water, 0.1 *N* NaOH ,and finally water until the filtrate was neutral. This residue and 0.5 gm of (Ethylenedinitrilo) tetraacetic acid disodium salt (EDTA) in 100 ml of water were autoclaved at 15 psi for 1 hr. The solution was cooled to room temperature, and the pH was adjusted to 2.0 with dilute hydrochloric acid before 0.50 gm of pepsin was added. The suspended sample was agitated intermittently for 72 hr, with adjustment of the pH when necessary. Then the residue was filtered and washed with acid, water, alkali, and water as before. This residue and 0.2 gm of EDTA in 100 ml of water were autoclaved as before, then cooled to room temperature, and the pH was adjusted to 8.0 with dilute sodium hydroxide. Pronase (0.20 gm) was added, and the digestion was continued for 72 hr, with adjustment of the pH when necessary. The residue was filtered and washed as before with acid, water, alkali, and water. The air-dried weight was 1.70 gm.

Gel Permeation Chromatography

A portion of this residue was solvent fractionated in 100 ml of 80 percent chloroform and 20 percent methanol, with intermittent stirring for 72 hr (Figure 3). The sample was centrifuged, and an aliquot of the soluble portion containing about 200 mg in 25 ml was fractionated on a 2.5 x 75 cm CPG-10-1000

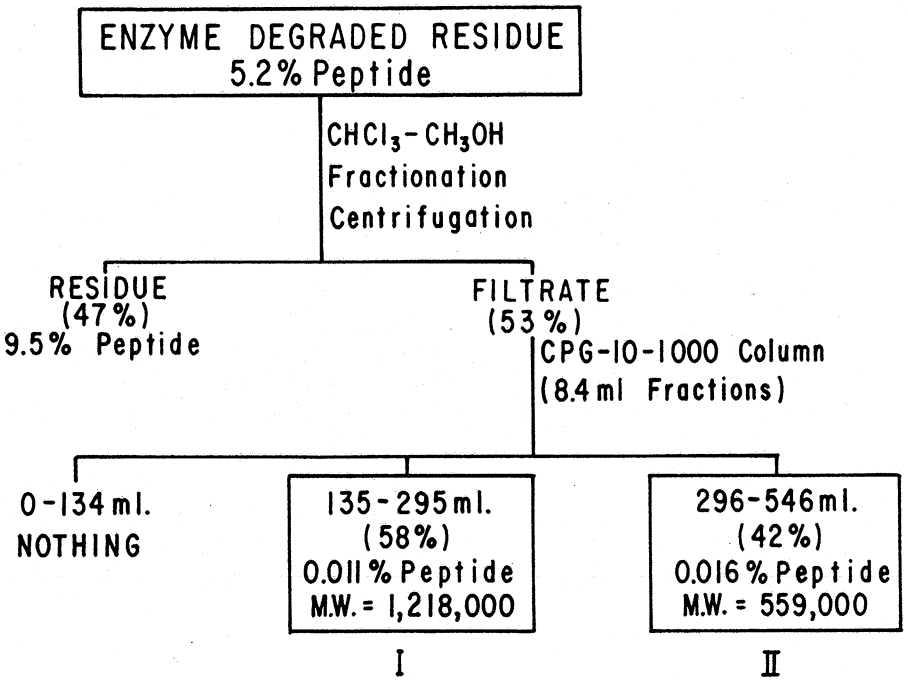

FIGURE 3.—Fractionation of enzyme degraded residue.

column with the same chloroform-methanol solvent as the eluant. This column has an operating range of 10^5 to 10^8 molecular weight. An upward flow was used and 8.4-ml fractions were collected in test tubes at the approximate rate of 1 ml/min. The eluants from the first five test tubes were combined and transferred to a 50-ml round bottom flask. The solvent was removed by evaporation under reduced pressure and ambient temperature to obtain any polymer present. The eluants from the succeeding test tubes were combined and evaporated in a similar manner. Only one peak was present, but this was split approximately in half on a weight basis (Figure 3). Analyses for molecular weight and peptide content were performed on both.

Viscosity Determinations for Molecular Weight Calculations

Several dilutions of the poly(methyl methacrylate) standards and of the

unknown polymer samples were used to determine the intrinsic viscosity necessary for the molecular weight calculation. The samples were dissolved in 2-butanone, and the specific viscosities were determined at 30°C in a Cannon-Manning semimicro viscometer. The data for the specific viscosities at various concentrations were extrapolated to zero concentration to obtain the intrinsic viscosity. The intrinsic viscosities thus obtained were used in the equation $[\eta] = KM^a$ to determine the molecular weight by use of literature values (6) for K and a.

Amino Acid Analyses

The samples were prepared for amino acid analysis by hydrolysis in 6 *N* hydrochloric acid solution for 24 hr under an atmosphere of nitrogen. The polymer residue was removed by filtration and washed several times with water. These washings were added to the filtrate, and the excess hydrogen chloride was then removed by repeated evaporations under reduced pressure with the intermittent addition of deionized water. This residue was then made up to volume with 0.1 *N* hydrochloric acid solution. The analyses were carried out on a Piez-Morris (7) type ion-exchange column with a continuous gradient-elution buffer. The results were calculated and tabulated with an IBM 1130 computer by use of programs developed at our laboratory.

Because of the small amount of sample remaining after the molecular weight studies, a modified method was used for hydrolyzing these samples in preparation for amino acid analyses. An aliquot of the polymer solution was transferred to a test tube, and, after the 2-butanone was evaporated, 2 ml of 6 *N* hydrochloric acid was added, and the tube was sealed under vacuum. Hydrolysis was carried out in an oven at 100°C for 24 hr, and the sample was prepared for the amino acid analyzer as previously described.

IR Spectra

The secondary standards of poly(methyl methacrylate) and the unknown polymer fractions in 2-butanone were applied dropwise to different sodium chloride crystals. Several applications were made to form a film, and the crystals were dried with an infrared lamp between applications. A Perkin-Elmer 137 spectrophotometer was used for the studies.

Results and Discussion

Previous papers (1–3) in this series have shown that it is possible to graft polymerize various acrylates and methacrylates onto chrome-tanned animal hides and skins. These studies reported the physical and chemical properties of the hides after graft polymerization with vinyl monomers, alone or in combinations. A number of redox systems were also investigated, and the optimum conditions obtained in those studies were used in the present work. Rao *et al.* (8, 9) isolated the grafted vinyl polymer chains by both acid and enzymatic hydrolysis of the

collagen backbone. Their graft copolymers were characterized by infrared spectra, electron microscopy, and detection of amino acid end groups through the use of ninhydrin solution.

Characterization of the Product of the Polymerization

The present study characterizes the polymer-peptide fragment obtained by enzymatic hydrolysis and solvent fractionation of the poly(methyl methacrylate) graft copolymer. Analysis of the ground, unextracted, graft copolymer showed that amino acids accounted for 42.3 percent of the material (calculated on a residue weight basis), and the ash content was 3.2 percent (Figure 1). The amino acid analysis is probably on the low side because of the interference of large amounts of bound polymer and homopolymer in the ground graft copolymer. The amino acid analyses of duplicate hydrolysates were in good agreement. A large amount of polymer residue remained after hydrolysis, and although this residue was washed several times with water, some of the proteinaceous material probably was physically trapped in the residue. The nitrogen analysis of the ground graft copolymer reinforces this theory. If we convert the nitrogen content (8.45 percent) to hide substance (8.45 x 5.62 = 47.49 percent), then the sum of the ash, bound polymer, homopolymer, and hide substance equals 99.3 percent. As described in the Experimental Section, the residue from enzymatic digestions was subjected to acid and base washes to eliminate the possibility of entrapping proteinaceous material in the graft copolymer.

Three 24-hr periods of Soxhlet extraction removed a total of 26.60 percent homopolymer. The homopolymer obtained from the first extraction had a molecular weight of about 600,000 and that of the second about 300,000–400,000 (Figure 1). Independent experiments have shown the viscosity molecular weight of a latex made under our polymerization conditions to be about 500,000. This latex polymer would not be expected to penetrate very deeply into the fine structure of the skin and thus should be readily extractable. The lower molecular weight polymer in the second extraction is probably homopolymer formed deeper in the skin structure near the sites where grafting actually takes place. This could be from true homopolymer formation or from a disproportionation of the growing polymer chain. Disproportionation is well known to be the usual termination process of a poly(methyl methacrylate) chain. The time-temperature effect of the hot solvent extraction process could also be involved in the formation of the lower molecular weight polymer. This effect is discussed in further detail later. The final extraction produced insufficient material for a molecular weight determination. These exhaustive extractions were carried out to avoid contaminating the isolated graft fraction with homopolymer fractions.

The percent bound polymer was calculated from Kjeldahl nitrogen determinations on the skin before and after grafting treatment with methyl methacrylate:

$$100 - \left(\frac{8.45}{16.43} \times 100 \right) - 26.60 = 21.97\%$$

where: 8.45 = percent nitrogen after grafting, 16.43 = percent nitrogen before grafting, and 26.60 = percent homopolymer.

Characterization of the Isolated Graft Copolymers

The grafted product was digested with enzymes as shown in Figure 2. In our previous study (4), we used only pepsin and pronase. We included collagenase in the present work in an effort to assure greater digestion of the collagen. It was not immediately apparent that the collagenase was helpful. However, it may have aided in the subsequent digestions, since the amino acid content of the isolated graft copolymers was reduced from 2.83 percent in the previous work (4) with poly(butyl acrylate) to about 0.01 percent in the present work. Because collagenase is not as effective on denatured collagen (10), the graft copolymer was not autoclaved prior to the collagenase digestion. Amino acid analysis of the residue after enzyme digestion showed that there was a peptide still bound to the graft, amounting to 5.2 percent of its weight. A portion of this residue was solvent fractionated in chloroform-methanol (80:20) (Figure 3). The insoluble part, after solvent fractionation, contained 9.5 percent amino acids on a weight basis as a peptide.

Results of Molecular Weight Studies of Graft Copolymers

The soluble portion eluted as a broad single peak when chromatographed on the CPG column. It was split in half on a weight basis to determine any differences in molecular weight and amino acid content. As expected, the molecular weight of the front-running component had the higher value. The viscosity molecular weight of the polymer in the first half of the peak was 1,218,000 and that in the second half was 559,000. By comparison, the average molecular weight of the graft copolymer isolated by 6 *N* hydrochloric acid hydrolysis for 2½ hr, rather than the enzyme degradation procedure, was about 850,000. This sample was not fractionated, but the results are within the limits of the enzyme degraded sample.

The amino acid analyses showed only a trace of aspartic acid, glycine, and alanine for each half of the peak, amounting to between 0.01 and 0.02 percent amino acids on a weight basis in the isolated material. This compares with 2.83 percent amino acids in the isolated poly(butyl acrylate) graft copolymer (4).

It was impossible to determine the molecular weight of the residue containing 9.5 percent amino acids because of its insolubility. This amount of peptide is apparently sufficient to prevent solubilization in the 2-butanone used for the viscosity measurements. However, it appears that the polymer portion has about the same molecular weight range as that of the soluble fraction, since the enzyme

attack should be random regarding the site of amino acid attack; the polymer is unaffected. Also, the poly(methyl methacrylate) homopolymers are soluble in chloroform-methanol; therefore, the degree of solubility of the copolymers should be determined by the size of the polypeptide portion.

The amino acids contribute very little to the molecular weight of the isolated graft copolymers. Where the average molecular weight of the polymer-peptide is 1,218,000, only about 134 of this (0.011 percent) is contributed by amino acids. When we divide this by the average residue molecular weight for the amino acids present (81.1), we find one or two amino acids plus the one that is the site of attachment to the polymer chain. This is probably impervious to acid hydrolysis and thus remains attached to the synthetic polymer molecule. Dividing the molecular weight of the polymer by the molecular weight of the monomer (100.11) indicates that there are about 12,180 monomer units in this polymer-peptide fragment.

Similarly, for the polymer-peptide with a molecular weight of 559,000, only about 89 (0.016 percent) is contributed by the amino acids. If this is divided by 81.1, the graft polymer is seen to contain one amino acid plus the one attached to the polymer chain. Dividing 559,000 by 100.11 shows the polymer-peptide fraction to contain 5,584 monomer units.

To test the accuracy of the viscosity determinations over a wide molecular weight range, several concentrations of the poly(methyl methacrylate) standards in 2-butanone were run under the same conditions as the unknown samples. The secondary standard from Aldrich Chemical Company had a molecular weight of 105,000 by their data, while the secondary standard from Polysciences, Inc., was stated to have a molecular weight of about one million $\pm$ 20 percent. Our results compare favorably and indicate that the test method as well as the literature values for K and a give satisfactory results.

The IR spectra of the polymer-peptide fragments confirm that they are principally poly(methyl methacrylate). Similar results were found by Rao *et al.* (9).

Results of Molecular Weight Studies of Extracted Homopolymers

Several experiments were performed to determine differences in the molecular weight of the extracted homopolymers depending on the temperature, solvent used, and length of time for extraction. Room temperature extractions with ethyl acetate or acetone gave homopolymers with molecular weights in the 800,000 to 850,000 range for both solvents. A portion of each of these extracts was refluxed by being boiled under conditions simulating a Soxhlet extraction for 24 hr. In each case, the molecular weights of the homopolymers were reduced by about 100,000. It is interesting to note that where the average molecular weight of the graft copolymer was about 940,000 after enzyme degradation, it was about 850,000 after acid hydrolysis by refluxing. Homopolymers obtained by 24-hr

Soxhlet extractions had molecular weights of about 600,000 when either ethyl acetate or acetone was used for the extraction. A second 24-hr Soxhlet extraction with either the same or alternate solvent gave homopolymers with molecular weights between 300,000 and 400,000. These experiments indicate that heat and length of time for extraction do tend to break down the homopolymers, resulting in lower average molecular weight values than actually formed. This phenomenon probably would occur with the graft copolymer. Since the poly(methyl methacrylate) chain normally terminates by disproportionation, the graft copolymers and homopolymers would not necessarily have to be all one length.

Summary

In summary, the combined evidence from the sequential enzyme digestions, chloroform-methanol solubility, CPG chromatography and IR spectra taken together, are consistent with grafting to the collagen molecule. Both the graft copolymer and homopolymer are polydisperse. Further studies are necessary, however, to elucidate the mechanism and point of attachment of the polymer to the collagen molecule. In future experiments we plan to use acid hydrolysis rather than enzymes to reduce the time required to degrade the collagen, but this can be used only for poly(methyl methacrylate) grafts, since the ester groups in poly(butyl acrylate) are labile.

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Received July 11, 1978.

Discussion

DR. WILLIAM C. PRENTISS (Rohm and Haas Company): Helen, thank you very much for that wonderful presentation.

Grafting experiments have been run with various vinyl monomers for many, many years. We have seen grafting to cellulose, grafting to wood — actually grafting to any number of things — and now we are beginning to get some in-

sights that elucidate the mechanism of grafting to collagen as a result of these very carefully done experiments that Helen Gruber has described for us this afternoon. They have been able to identify some of the amino acids connected to the hydrolyzed graft polymer. I'm going to pose a question to Helen: From this kind of information, can you get any indication whatsoever as to the site of grafting?

Ms. GRUBER: So far we haven't been able to get any indication as to the actual amino acid residue that is attached to the collagen molecule. We have done some other studies using poly(butyl acrylate) and we've found additional amino acids in the sequence attached to the polymer molecule. However, we do hope in the future that we will be able to identify the site.

DR. PRENTISS: I have an additional question. In your hydrolysis you used three different enzymes to get the breakdown of the system, and yet in these various digestions that you go through, you end up with a fraction that has a very small quantity of attached amino acid, and a residue that is relatively high, that is a low of 0.01 from a net range of 0.99 on the other. Can you comment about the activity of the enzyme digestion with the graft polymers related to the same digestion in the ungrafted state?

Ms. GRUBER: The enzymes that we used were pepsin, pronase, and collagenase. They are nonspecific enzymes. They attack the protein in the center of the collagen molecule. We expect that they would attack the same sites in the graft copolymer as they would in the collagen molecule.

DR. PRENTISS: Thank you. Any other questions? Dr. Buechler.

DR. PETER BUECHLER (PPG Industries): I would like to say that the evidence seems extremely conclusive that you indeed have grafting here, and the point is that you have achieved a breakdown, and you had to go to great lengths to do it, which seems to show that there would be a lot of preservative action by the modification of the protein molecule introduced by the polymerization. Would you care to comment on that point as to how difficult it was, and therefore, as to whether you may have here a method of tanning *per se?* Tanning is defined by some people principally in terms of its preservative action for hides. Enzymes are supposed to fit substrate protein by a lock-and-key mechanism. If we regard the enzyme as the "key," by grafting you may be changing the conformation of the collagen "lock" so the "key" won't fit.

Ms. GRUBER: Well, there probably are easier methods of degrading the collagen molecule, but what we wanted to do was to isolate a specific polymer attached to a small peptide portion. So that's why we went through this more or less elaborate fractionation procedure. Also, previous studies do not indicate tanning *per se,* and in fact, resistance to enzyme degradation is only one aspect of practical tannage.

DR. BUECHLER: What happens if you just use solvent extraction alone, which should remove the homopolymer? How much residue would you get? Did you try this?

MS. GRUBER: Yes, we had quite a bit of residue, because all of the collagen remains after solvent extraction. As a matter of fact, we would have more collagen than polymer because we have approximately 22 percent polymer bound to the collagen molecule. So we really need to degrade it to get rid of most of the collagen molecule before we can do any characterization studies.